

A Canine Liver Fibrosis Model to Develop a Therapy for Liver Cirrhosis Using Cultured Bone Marrow–Derived Cells

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We have been developing a therapy for liver cirrhosis using cultured autologous bone marrow–derived mesenchymal stem cells (BMSCs). Before human clinical trials can be considered, the safety and efficacy of BMSC infusion in medium to large animals must be confirmed; thus, we developed a canine liver fibrosis model. A small amount of bone marrow fluid was aspirated from the canine humerus to assess the characteristics of BMSCs. We implanted a venous catheter in the stomach and a subcutaneous infusion port in the back of the neck of each canine. Repeated injection of CCl₄ through the catheter was performed to induce liver cirrhosis. After 10 weeks of CCl₄ injection, eight canines were equally divided into two groups: no cell infusion (control group) and autologous BMSC infusion through the peripheral vein (BMSC group). A variety of assays were carried out before and 4 weeks after the infusion. The area of liver fibrosis stained with sirius red was significantly reduced in the BMSC group 4 weeks after BMSC infusion, consistent with a significantly shortened half-life of indocyanine green and improved liver function. *Conclusion:* We established a useful canine liver fibrosis model and confirmed that cultured autologous BMSC infusion improved liver fibrosis without adverse effects. (*Hepatology Communications* 2017;1:691-703)

Liver cirrhosis is the end stage of progressive hepatic fibrosis and is characterized by distorted liver architecture and regenerative nodule formation. Many studies have shown evidence of the therapeutic effects of bone marrow (BM) cells in chronic liver diseases with liver fibrosis,⁽¹⁻³⁾ and antifibrotic therapy is now an important approach to the treatment of liver cirrhosis.⁽⁴⁾

We previously developed a green fluorescent protein/CCl₄ murine model and reported that in an environment of chronic inflammation, donor-derived BM cells degrade existing liver fibrosis by producing matrix metalloproteinase-9 and other collagenases, leading to

significant improvements in liver fibrosis, hepatic functions, and survival outcome.⁽⁵⁻⁸⁾ In addition, based on these results, a clinical trial of autologous BM cell infusion (ABMi) therapy for liver cirrhosis began in 2003,⁽⁹⁾ and a multicenter clinical trial (Liver Regeneration with Cell Transplantation study) began in 2005.⁽¹⁰⁾ Kim et al. used ABMi therapy to treat patients with decompensated hepatitis B virus–related liver cirrhosis and reported elevated serum albumin levels and improved Child-Pugh scores.⁽¹¹⁾ Saito et al. also reported the efficacy and safety of ABMi therapy for alcoholic liver cirrhosis.⁽¹²⁾ Although ABMi therapy has favorable effects in patients with liver cirrhosis,

Abbreviations: ABMi, autologous bone marrow cell infusion; BM, bone marrow; BMSC, bone marrow–derived mesenchymal stem cell; BW, body weight; CD, cluster of differentiation; CE-CT, contrast-enhanced computed tomography; COL1A2, collagen type 1 alpha 2; COL3, collagen type 3 alpha 1; DMEM, Dulbecco's modified Eagle medium; EDNRA, endothelin receptor type A; EDNRB, endothelin receptor type B; FDP, fibrin degradation products; ICG, indocyanine green; MSC, mesenchymal stem cell; PSS, portosystemic shunting; TIMP-1, tissue inhibitor of metalloproteinase 1.

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it requires BM aspiration under general anesthesia; and some patients are excluded due to their poor liver or cardiopulmonary functions. As part of our efforts to expand the applicability of ABM_i therapy, we developed a less invasive method for liver regeneration therapy using cultured autologous BM-derived mesenchymal stem cells (BMSCs) from small amounts of BM fluid aspirated under local anesthesia. Recently, the therapeutic potential of BMSCs for the treatment of liver injury has been evaluated, and several studies have provided experimental evidence suggesting that transplantation of BMSCs can sustain liver function after liver injury.⁽¹³⁾ *In vitro* research has shown that BMSCs induce apoptosis and suppress collagen synthesis in hepatic stellate cells.⁽¹⁴⁾ Moreover, *in vivo* studies have demonstrated the antifibrotic and anti-inflammatory effects of BMSCs injected through a peripheral vein.^(15,16) Before human clinical trials can be considered, the safety and efficacy of cultured autologous BMSC infusion in medium to large animals must be confirmed. In this regard, the similarities in anatomy and pathogenesis make canines a very attractive model for research on BMSCs for application in humans as they facilitate a more detailed evaluation of therapeutic effects compared with rodent models.⁽¹⁷⁾

Here, we developed a canine liver fibrosis model to demonstrate the safety and efficacy of infusion of cultured autologous BMSCs for the treatment of cirrhosis.

Materials and Methods

ANIMALS AND ETHICS

Sixteen beagles (1-2 years old, 8 male and 8 female) were used in this study. The canines were housed in

the animal facility at Yamaguchi University and treated in accordance with the university's animal care guidelines. The study was approved by our Institutional Ethics Committee (approval no. 21-033).

CATHETER IMPLANTATION

An intravenous catheter was inserted for administration of a loading dose of propofol (7 mg/kg body weight [BW]; 1% Propofol inj. Maruishi; Maruishi Pharmaceutical Co. Ltd., Osaka, Japan). An endobronchial tube was also employed. All canines received positive pressure ventilation using an Apollo anesthetic machine (Dräger Medical Japan). Anesthesia was maintained with isoflurane (DS Pharma Animal Health Co., Ltd., Osaka, Japan) in oxygen. The end-tidal isoflurane concentration was monitored and maintained between 1.4% and 2.8%. Intravenous buprenorphine (Lepetan 0.2 mg; Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan) was administered at a dose of 10 μ g/kg BW to provide effective pain relief.

All catheter implantations were performed under endoscopic view. The stomach was directly punctured with an 18-gauge Teflon IV catheter (6-French P-U catheter; Toray, Tokyo, Japan) through a small incision. The catheter tip was positioned in the stomach by the Seldinger technique. An infusion port (P-U Celsite port; Toray) was placed in a subcutaneous pocket created on the back. An indwelling catheter was inserted subcutaneously and connected to the infusion port. Intramuscular buprenorphine at 10 μ g/kg BW and subcutaneous cefovecin (Convenia; Zoetis Japan, Tokyo, Japan) at 8 mg/kg BW were given at the end of the procedure for postoperative analgesia and to prevent infections, respectively.

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CANINE BMSC ISOLATION AND CULTURE CONDITIONS

About 2 mL BM fluid was aspirated from the proximal humerus using a 16-gauge biopsy needle (Angiotech, Gainesville, FL) after catheter implantation. The BM fluid was seeded into T-75 flasks (Life Technologies, Grand Island, NY) and cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies) and gentamicin (100 $\mu\text{g}/\text{mL}$; Life Technologies) in a 5% CO_2 incubator at 37°C. After 2 days of incubation, nonadherent cells were removed during medium replacement. The culture medium was changed every 2 days, and cells were cultured for around 3 weeks with two or three cell passages to obtain BMSCs for this study.

FLOW-CYTOMETRIC ANALYSIS OF CULTURED CANINE BMSC PREPARATIONS

Phenotypic characterization of canine BMSCs was performed as described.⁽¹⁸⁾ Adherent cells were dissociated with 0.05% trypsin-ethylenediaminetetraacetic acid (Life Technologies) and resuspended in DMEM containing 10% fetal bovine serum. The cells were washed once with phosphate-buffered saline (Life Technologies), and after centrifugation the phosphate-buffered saline was removed and replaced with phosphate-buffered saline containing 2% canine serum (AbD Serotec, Oxford, UK). The cells were placed on ice for 20 minutes, followed by incubation for a further 20 minutes on ice with monoclonal antibodies against cluster of differentiation 11b (CD11b; AbD Serotec), CD29-PE (Abcam, Cambridge, UK), CD44-APC (Biolegend, San Diego, CA), CD45-eFlour (ebioscience, San Diego, CA), and CD90-APC (ebioscience). The CD11b antibody was detected using goat polyclonal secondary antibody to mouse immunoglobulin G heavy and light chains (DyLight 488; Abcam). Isotype-identical antibodies were used as controls. Flow-cytometric analyses were performed using the Gallios system (Beckman Coulter, Danvers, MA). Propidium iodide (Sigma-Aldrich, St. Louis, MO) was used to exclude dead cells from analyses. Each sample was assessed at least in triplicate. Data were analyzed using Kaluza software (Beckman Coulter).

IN VITRO DIFFERENTIATION OF CULTURED BMSCs

To confirm differentiation potential, cells were grown in osteogenic and adipogenic canine differentiation media (Cell Applications, San Diego, CA) in accordance with the manufacturer's instructions. To promote osteogenesis, the cells were incubated in DMEM supplemented with 10 mM β -glycerol phosphate (Sigma-Aldrich), 0.05 mM ascorbate-2-phosphate (Sigma-Aldrich), and 100 nM dexamethasone (Sigma-Aldrich). The culture medium was changed three times per week for up to 2 weeks. To detect deposition of bone mineral, the cells were fixed with methanol for 10 minutes at room temperature and stained with alizarin red (AppliChem, Darmstadt, Germany) at pH 4.0 for 5 minutes at room temperature. For adipogenesis, cultured cells were incubated in adipogenic medium that included DMEM supplemented with 60 mM indomethacin (Sigma-Aldrich), 0.5 mM hydrocortisone (Sigma-Aldrich), and 0.5 mM isobutylmethylxanthine (Sigma-Aldrich). The culture medium was changed three times per week for up to 2 weeks. The cells then were fixed in methanol for 45 minutes and stained with oil red O (Sigma-Aldrich) for detection of lipid accumulation.

EXPERIMENTAL MODEL (FIBROSIS INDUCTION)

CCl_4 (Wako, Osaka, Japan) was diluted 1:1 in corn oil and repeatedly injected for 10 weeks using the implanted catheter (high-dose period, 1.0 mL/kg BW once a week and 0.5 mL/kg BW once a week for 6 weeks; low-dose period, 0.25 mL/kg BW twice a week for 8 weeks) to induce liver fibrosis (Fig. 1). CCl_4 was injected on the first day and the fourth day of each week.

EXPERIMENTAL GROUPS

After 10 weeks of CCl_4 injections, 16 canines were divided equally into two groups of eight. This point was defined as "0W."

Subsequently, the eight canines in the control group received low-dose CCl_4 injection for a further 4 weeks. The eight canines in the BMSC group were infused with cultured autologous BMSCs ($4 \times 10^5/\text{kg}$) through a peripheral vein, and low-dose CCl_4 injection was continued for 4 more weeks.

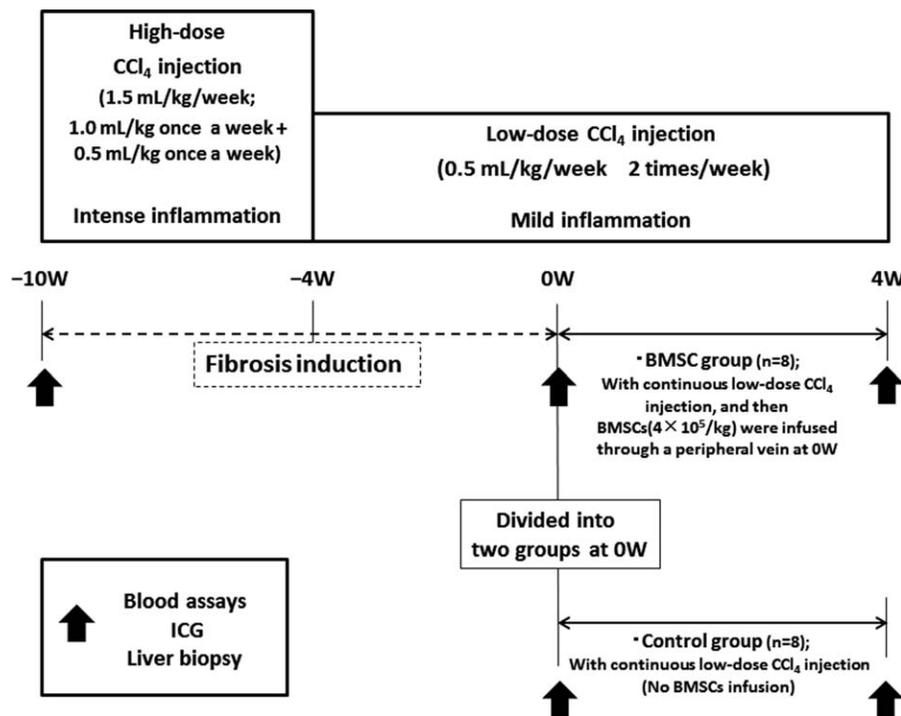


FIG. 1. Experimental protocol. Canines were given repeated CCl_4 injections through the implanted catheter for 14 weeks (high-dose period, 1.0 mL/kg body weight once a week and 0.5 mL/kg body weight once a week for 6 weeks; low-dose period, 0.25 mL/kg body weight twice a week for 8 weeks). CCl_4 was injected on the first day and the fourth day of each week. At 0 weeks (0W; i.e., after 10 weeks of CCl_4 injection), canines were divided into two groups. In the control group, low-dose CCl_4 injection was continued for a further 4 weeks without BMSC infusion. In the BMSC group, cultured autologous canine BMSCs ($4 \times 10^5/\text{kg}$) were infused through a peripheral vein, and low-dose CCl_4 injection was continued for 4 more weeks. In both groups, blood assays, ICG testing, and liver biopsy under ultrasonography were carried out at -10W , 0W , and 4W , as indicated by the arrows.

Blood examinations, ultrasonography-guided liver biopsies, and indocyanine green (ICG) tests were carried out before and 10 and 14 weeks after CCl_4 injection (i.e., at -10W , 0W , and 4W) (Fig. 1). For safety evaluation, oxygen saturation, pulse rates, and general condition were monitored before and after BMSC infusion. Furthermore, chest-abdominal contrast-enhanced computed tomography (CE-CT) was performed 30 minutes after BMSC infusion to evaluate the presence of pulmonary embolism and portosystemic shunting (PSS).

EVALUATION OF DOSAGE VARIANCE SAFETY

BMSCs were increased to 1.2×10^6 cells/kg and infused through a peripheral vein into a liver fibrosis-induced canine to test dosage variance ($n = 1$). Oxygen saturation, pulse rates, and general condition

were monitored before and after BMSC infusion. Blood parameters were measured before BMSC infusion and at 1 and 3 days after infusion. Seven days after BMSC infusion, we sacrificed the canine to evaluate the presence of lung embolization by histological analysis after collecting blood samples.

LABORATORY TESTS

Serum albumin, alanine aminotransferase, aspartate aminotransferase, bilirubin, prothrombin time, anti-thrombin 3 activity, and fibrin degradation products (FDP) were measured.

ICG TEST

ICG (Diadnogreen Inj.; Daiichi Sankyo Co. Ltd., Tokyo, Japan) was prepared immediately prior to administration. Following the collection of blood for

the zero-time sample, 0.1 mg ICG/kg was administered, and blood was collected 5, 15, and 30 minutes later. Serum was harvested from blood samples and analyzed for ICG content using an ICG meter (Fuchu Giken, Inc., Tokyo, Japan). The plasma half-life was calculated for each substance using semilog paper. The difference in the half-life of ICG, Δ ICG (minutes), was calculated using the following formula: Δ ICG = (half-life of ICG at 4W) – (half-life of ICG at 0W).

CE-CT IMAGING

CE-CT examinations were carried out using an eight-detector row CT system (ECLOS 8; Hitachi Medical Corp., Tokyo, Japan). All canines were placed in ventral recumbency. All CT scans were performed during apnea under anesthesia. Canines were given 2 mL/kg iopamidol with an iodine concentration of 370 mg/mL (Oiparomin 370; Fuji Pharmaceutical Co., Toyama, Japan) as intravenous contrast medium.

LIVER BIOPSY

After intramuscular injection of medetomidine (Dorbene; Kyoritsu Seiyaku Co., Tokyo, Japan) at 20 μ g/kg, canines were placed in the left lateral decubitus position. Local anesthetic (0.5% lidocaine; Pfizer) was injected into a small area of skin and tissues over part of the liver, and ultrasonography-guided liver biopsies were carried out using a 16-gauge biopsy needle (Aragon Medical Devices, Plano, TX). Some samples were fixed in 4% paraformaldehyde overnight and used for histological staining, and others were stored at -80°C for microarray analysis and real-time quantitative PCR.

HISTOLOGICAL STAINING

Paraffin-embedded liver samples were sectioned (3 μ m) and stained with sirius red as described.⁽¹⁸⁾

HISTOMORPHOMETRY

Histomorphometry was performed using an imaging system coupled to a fluorescence microscope (Biorevo BZ9000; Keyence, Osaka, Japan). The fibrotic area was calculated as the percent of the sirius red-stained area of the total sample using a BZ Analyzer II (Keyence). Vessels stained with sirius red were excluded from the calculation. We defined the percentage of the fibrotic area as the fibrosis level. The difference in the fibrotic area, Δ fibrosis level (%), was calculated using

the following formula: Δ fibrosis level = (fibrosis level at 4W) – (fibrosis level [%] at 0W).

DNA MICROARRAY ANALYSIS

We generated the expression profiles of the canine livers using the DNA-chip system (Agilent Technologies, Santa Clara, CA) and analyzed the expression patterns using IPA software (Ingenuity Systems, Redwood City, CA).

REAL-TIME QUANTITATIVE PCR ANALYSIS

Total RNA extraction was performed using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany). For complementary DNA synthesis, TaqMan reverse transcription reagents were used as described in the manufacturer's manual (Roche Diagnostics, Indianapolis, IN). Variations in gene expression were analyzed using a Step One Plus real-time PCR system (Life Technologies) with SYBR green. Relative quantification of gene expression was performed using ribosomal protein 18 as an internal control. The primers used were as follows: canine collagen, type1, alpha2 (*COL1A2*), sense (5'-CCCAGCCAAGAAGTGGTACAGAA-3') and antisense (5'-CGCATGAAGGCGAGTTGAGTAG-3'); canine collagen, type3, alpha1 (*COL3*), sense (5'-CATCTCGGCACAGCAGCAA-3') and antisense (5'-CAGATCCTGAGTCACAGACGCATA-3'); canine tissue inhibitor of metalloproteinase 1 (*TIMP-1*), sense (5'-TTCACCAAGACCTATGCTGCTGCTG-3') and antisense (5'-AGTTGCATATCCCTGGCTCTC-3'); canine endothelin receptor type A (*EDNRA*), sense (5'-ATCGGTATTAAC TTGGCAACCATGA-3') and antisense (5'-GGACTGGTAACAGCAACAGCAGAG-3'); canine endothelin receptor type B (*EDNRB*), sense (5'-CATGCGA AACGGCCCTAATATC-3') and antisense (5'-GGCACCAGCTTACACATCTCCA-3'); and canine ribosomal protein 18, (5'-ATAGCCTTTGCCATCAGCAATTAA-3') and antisense (5'-TTGGTGAGATCGATGTCTGCTTTC-3').

STATISTICAL ANALYSIS

Data were analyzed using the Student *t* test and paired *t* test. Values of *P* < 0.05 were considered statistically significant. Data are presented as the mean \pm standard deviation.

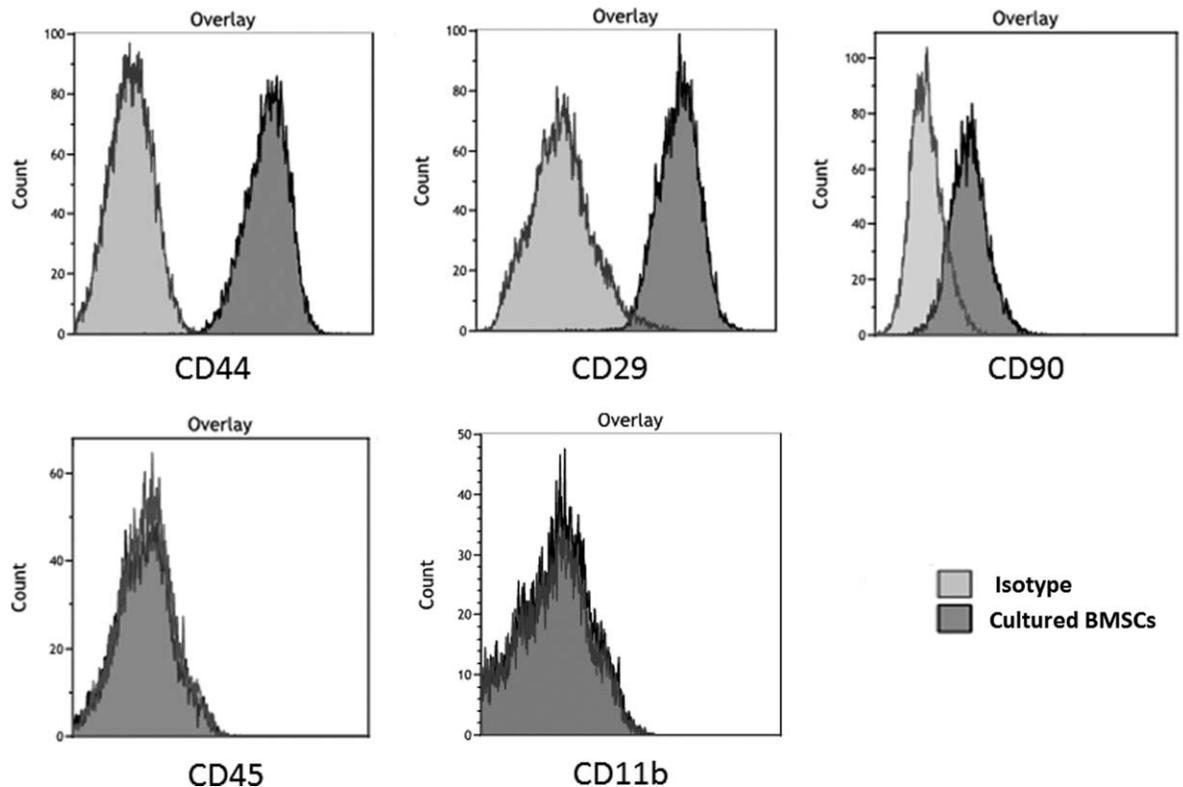


FIG. 2. Flow-cytometric analysis of BMSCs. BMSCs used for infusion did not express CD45 or CD11b and were positive for CD44, CD29, and CD90.

Results

CELLULAR CHARACTERIZATION

The cells used in this study adhered to plastic and were homogeneously distributed with a fibroblastoid shape. Flow-cytometric analyses showed that the cultured canine BMSCs were positive for CD29, CD44, and CD90 and negative for the pan-leukocyte marker CD45 and the monocyte/macrophage marker CD11b (Fig. 2). The cells also had the potential to differentiate into adipogenic and osteogenic lineages, consistent with a typical BMSC phenotype (Fig. 3).

ASSESSMENT OF FIBROSIS AREA

Bridging fibrosis was confirmed at 0W with sirius red staining, and we observed pseudolobules in some samples. The fibrosis level was increased in the control group from $10.9 \pm 4.2\%$ at 0W to $11.6 \pm 4.1\%$ at 4W. In contrast, a significant decrease was observed in the BMSC group ($9.2 \pm 2.9\%$ at 0W to $7.2 \pm 3.5\%$ at

4W, $P < 0.05$) (Fig. 4A,B). Moreover, the fibrosis level was significantly lower at 4W in the BMSC group compared with 4W in the control group ($P < 0.05$) (Fig. 4C). The Δ fibrosis level was $0.7 \pm 0.8\%$ in the control group and $-2.1 \pm 1.1\%$ in the BMSC group ($P < 0.05$) (Fig. 4D).

ICG TEST RESULTS

The half-life of ICG was prolonged in the control group (14.9 ± 4.1 minutes at 0W, 16.8 ± 5.3 minutes at 4W) and was slightly shorter in the BMSC group (13.4 ± 2.2 minutes at 0W, 12.4 ± 2.2 minutes at 4W). Δ ICG was -1.0 ± 0.3 minutes in the BMSC group and 1.9 ± 0.7 minutes in the control group ($P < 0.05$) (Fig. 4E,F).

BIOCHEMICAL RESULTS

The alanine aminotransferase, albumin, and anti-thrombin 3 levels significantly improved in the BMSC group at 4W compared with the control group at 4W.

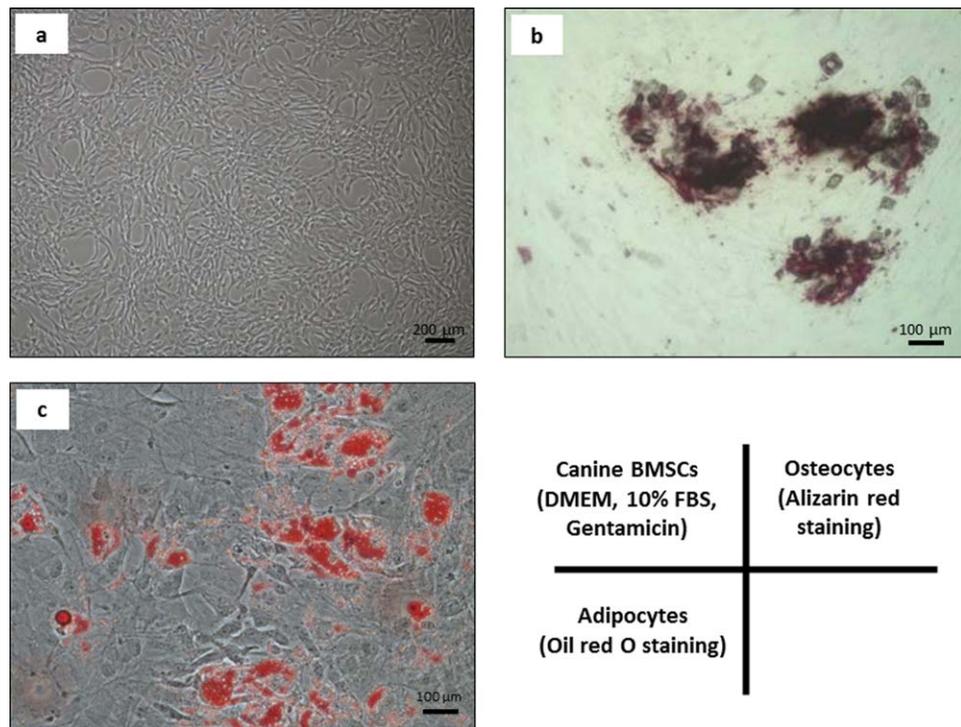


FIG. 3. Canine BMSC characterization. (A) BMSCs adhered to plastic and had fibroblast-like morphology. The differentiation potential of BMSCs was evaluated in osteogenic and adipogenic induction medium and analyzed by (B) alizarin red and (C) oil red O staining. Abbreviation: FBS, fetal bovine serum.

Aspartate aminotransferase, bilirubin, prothrombin time, and FDP in the control group and the BMSC group were not significantly different (Table 1).

SAFETY EVALUATIONS

CE-CT revealed no sign of fatal thrombosis in lung arteries and the absence of major PSS (Fig. 5A). We did not observe oxygen desaturation, a remarkable change in pulse rates, or a decline in the general condition after BMSC infusion in any canines including the canine for the test of dosage variance. In the canine that was infused with a higher number of BMSCs (1.2×10^6 cells/kg), elevation in FDP levels was not observed as follows: before, $1.4 \mu\text{g/mL}$; day 1, $2.4 \mu\text{g/mL}$; day 3, $1.9 \mu\text{g/mL}$; day 7, $3.3 \mu\text{g/mL}$. Moreover, lung embolization was not observed in the lung tissues following hematoxylin and eosin staining (Fig. 5B).

EXPRESSION OF LIVER FIBROSIS-RELATED GENES

Using K-means clustering analysis, changes in gene expression patterns were compared between the BMSC group before and after 4 weeks of BMSC administration (BMSC-0W and BMSC-4W, respectively) and

the control group at the same time points (Cont-0W and Cont-4W, respectively). The expression of certain gene clusters was significantly decreased in the BMSC-4W group. Further analysis showed that these clusters included the following genes related to toxic function that are associated with liver fibrosis: *EDNRA* (log ratio, -0.3 ; $P < 0.01$), *EDNRB* (log ratio, -0.6 ; $P < 0.05$), and *COL1A2* (log ratio, -0.8 ; $P < 0.05$). Figure 6A shows the changes in expression of these genes in the BMSC and control groups.

Furthermore, we analyzed the mRNA expression of *COL1A2*, *COL3*, *TIMP-1*, *EDNRA*, and *EDNRB* in the liver at -10W , 0W , and 4W . The expression of all genes was much higher at 0W than at -10W . *COL1A2*, *COL3*, and *EDNRA* expression levels were significantly higher at 4W compared with 0W in the control group. At 4W , *COL1A2*, *COL3*, *TIMP-1*, *EDNRA*, and *EDNRB* expression levels were significantly lower in the BMSC group compared with the control group (Fig. 6B).

Discussion

In this study, we developed a model for canine liver fibrosis and then confirmed the safety and effects of

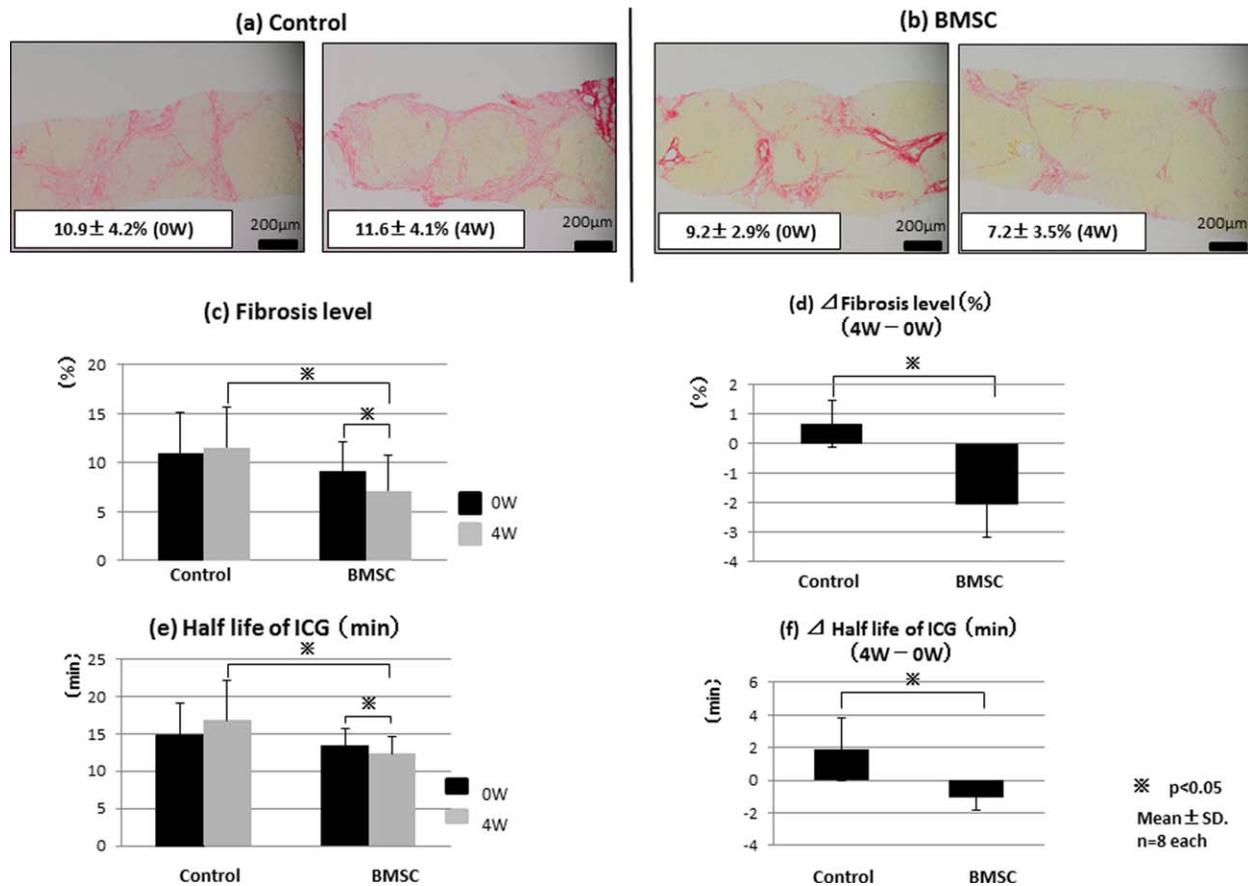


FIG. 4. Liver fibrosis assessed with sirius red staining and the therapeutic effects of transused BMSCs in the CCl₄-induced canine liver fibrosis model. (A) The fibrosis level was increased in the control group from 10.9 ± 4.2% at 0W to 11.6 ± 4.1% at 4W. (B) In contrast, a decrease was observed in the BMSC group (9.2 ± 2.9% at 0W to 7.2 ± 3.5% at 4W). (C) The fibrosis level was significantly lower at 4W in the BMSC group (7.2 ± 3.5%) compared with 0W in the same group (9.2 ± 2.9%, *P* < 0.05) and 4W in the control group (11.6 ± 4.1%, *P* < 0.05). (D) The Δfibrosis level was 0.7 ± 0.8% in the control group and -2.1 ± 1.1% in the BMSC group (*P* < 0.05). (E) The half-life of ICG was significantly shorter at 4W in the BMSC group (12.4 ± 2.2 minutes) compared with 0W in the same group (13.4 ± 2.2 minutes, *P* < 0.05) and 4W in the control group (16.8 ± 5.3 min, *P* < 0.05). (F) ΔICG was -1.0 ± 0.3 minutes in the BMSC group and 1.9 ± 0.7 minutes in the control group (*P* < 0.05). All error bars represent the standard deviation of the mean.

TABLE 1. CLINICAL LABORATORY TESTS

	Normal Range	Control (n = 8)		BMSC (n = 8)	
		0W	4W	0W	4W
AST (U/L)	18-53	49.3 ± 26.0	71.3 ± 36.4	48.0 ± 26.3	39.7 ± 16.3
ALT (U/L)	20-109	307.3 ± 197.4	522.3 ± 344.8	134.7 ± 61.2	142.3 ± 89.6*
Bil (mg/dL)	0.0-0.1	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
Alb (g/dL)	2.5-3.5	2.5 ± 0.2	2.5 ± 0.2	2.7 ± 0.2	2.8 ± 0.1*
PT (seconds)	6.1-9.6	8.4 ± 1.0	8.5 ± 1.2	7.5 ± 0.5	7.4 ± 0.9
AT3 (%)	116-161	111.0 ± 17.1	102.4 ± 14.0	111.1 ± 12.2	118.6 ± 10.5*
FDP (μg/mL)	<5.0	2.1 ± 1.4	2.9 ± 1.5	2.0 ± 0.7	3.9 ± 4.3

Data are the mean ± standard deviation.

**P* < 0.05 versus 4W in control.

Abbreviations: Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AT3, antithrombin3; Bil, bilirubin; PT, prothrombin time.

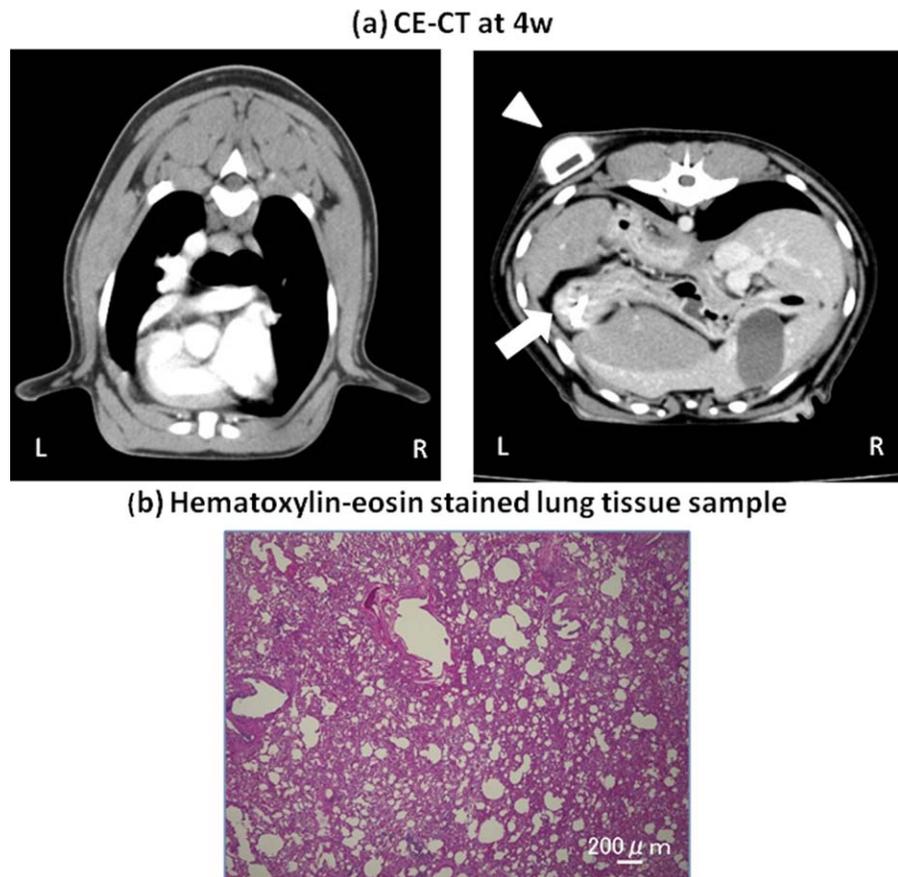


FIG. 5. CE-CT at 4W and a photomicrograph of lung tissue samples. (A) There were no signs of pulmonary embolism or PSS in the CCl_4 -induced canine liver fibrosis model. Arrowhead indicates the infusion port placed in a subcutaneous pocket on the back. Arrow indicates the catheter tip positioned in the stomach. (B) Lung embolization was not observed in the lung tissue samples following hematoxylin and eosin staining.

infusion of cultured autologous BMSCs through a peripheral vein. The cells infused in this model had salient characteristics of mesenchymal stem cells (MSCs)⁽¹⁹⁾; such as adherence to plastic; fibroblast-like morphology; expression of CD29, CD44, and CD90; and absence of CD11b and CD45 expression (Fig. 2). In many studies in humans and canines, CD29, CD44, and CD90 are considered cell-surface markers for MSCs,⁽²⁰⁻²²⁾ whereas CD34 and CD45 are considered negative surface markers.^(19,23-25) Our results showed that the expression of CD90 was lower than that of other markers, similar to the report by Takemitsu et al.,⁽²⁶⁾ who suggested that the decline in CD90 expression may be related to differences in species or passage number.

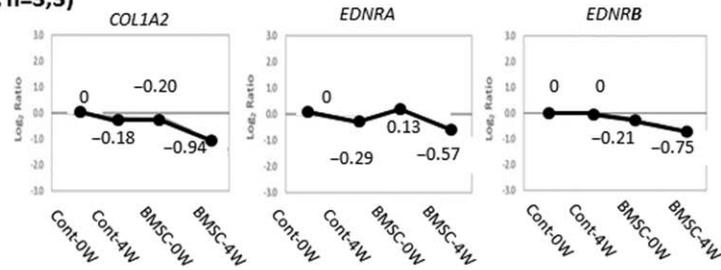
Initially, we repeatedly administered CCl_4 orally (0.1 mL/kg BW, 5 times/week) to canines to induce liver fibrosis. Administration of CCl_4 for over 20 weeks produced liver fibrosis, but we encountered two problems with this procedure. First, the canines could

not tolerate much CCl_4 orally due to the pungent smell and unappealing taste. Second, it was difficult to administer CCl_4 reliably because some canines strongly resisted CCl_4 administration by the mouth. Therefore, it is possible that the extended duration of fibrosis induction produced some heterogeneity in liver fibrosis among canines. Boothe et al. developed dimethylnitrosamine-induced hepatic disease in canines. However, homogeneous liver disease was difficult to establish, and this group observed great variability in the duration of dimethylnitrosamine administration.⁽²⁷⁾ Wong et al.⁽²⁸⁾ also tried to induce canine liver fibrosis with elevation of portal pressure by repeated intraperitoneal injections of CCl_4 for 12-24 weeks. In these studies,^(27,28) over 20 weeks were needed to induce liver disease in some canines.

We therefore developed another canine liver fibrosis model using an implanted catheter to overcome these problems. Using this system, we established an intense inflammation period for 6 weeks to induce a severe

(a) Analyses of microarray data (Control, BMSC; n=3,3)

Gene Name	Log ₂ Ratio	p-value
Collagen, type I, alpha2 (COL1A2)	-0.80	<0.05
Endothelin receptor typeA (EDNRA)	-0.30	<0.01
Endothelin receptor typeB (EDNRB)	-0.60	<0.05



(b) Real time quantitative PCR (Control, BMSC: n=8,6; 2samples from each biopsy tissue)

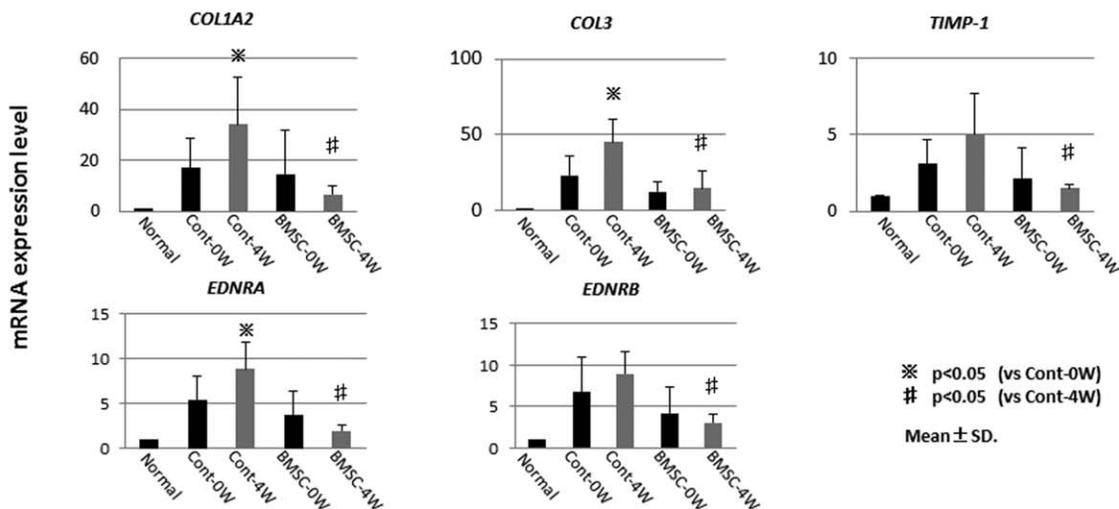


FIG. 6. Expression of liver fibrosis-related genes. (A) Microarray analysis (control n = 3, BMSC n = 3). Expression of *COL1A2*, *EDNRA*, and *EDNRB* at 0W was significantly decreased compared with 4W in the BMSC group. Data for Cont-0W, Cont-4W, BMSC-0W, and BMSC-4W were plotted as log₂ ratios with Cont-0W as the denominator. (B) Real-time quantitative PCR (control n = 8, BMSC n = 6; two samples from each biopsy tissue). *COL1A2*, *COL3*, and *EDNRA* expression levels were significantly higher at 4W compared with 0W in the control group ($P < 0.05$). *COL1A2*, *COL3*, *TIMP-1*, *EDNRA*, and *EDNRB* expression was significantly lower at 4W in the BMSC group compared with 4W in the control group ($P < 0.05$). Data show the mean \pm standard deviation.

liver disorder; and after that period, we injected a lower dose of CCl₄ to maintain the liver inflammation. Bridging fibrosis was confirmed in all canine liver samples after 10 weeks of CCl₄ administration. However, some differences were noted in the severity of liver fibrosis. Cytochrome 2E1 is the major factor involved in CCl₄-induced hepatotoxicity. Lankford et al. reported a variant allele of cytochrome 2E1 in 19% of beagles.⁽²⁹⁾ Thus, some canines in this experiment may have expressed the cytochrome 2E1 genetic variant, and these animals may be relatively resistant to CCl₄-induced hepatotoxicity. In the future, littermates should be used to reduce experimental heterogeneity.

For safety evaluation, we measured oxygen saturation, blood parameters, and CE-CT before and after BMSC infusion. Although some researchers have

concerns regarding the procoagulation capacity of cultured BMSCs, there was no fatal thrombosis in lung arteries using CE-CT after the infusion. Performing CE-CT with small animals such as rodents is difficult, and therefore, medium-sized animals such as canines are extremely useful for this imaging test. Oxygen desaturation, elevation of FDP, and decline in the general condition of the canines also did not occur after BMSC infusion. In the test of dosage variance, a significant elevation of FDP was not observed. Overall, these evaluations indicated that BMSC infusion through a peripheral vein is safe.

We assessed the half-life of ICG to evaluate liver function. As early as 1961, the ICG elimination rate was widely used to measure liver function and hepatic blood flow.⁽³⁰⁾ ICG is extracted nearly exclusively by

hepatic parenchymal cells and is excreted almost entirely into the bile without enterohepatic circulation.⁽³¹⁾ Boothe et al. proved the diagnostic benefits of using ICG disposition kinetics as a method of evaluating hepatic function in canines with progressive liver disease.⁽²⁷⁾ Other investigators provided evidence that elimination of ICG may not be sensitive to changes in blood flow induced by hepatic disease.^(32,33)

In the case of obstructive biliary diseases or vascular shunt of hepatic circulation, the results of the ICG test may be worse than the true functional reserve.^(34,35) Howe et al. developed an experimental canine model of dimethylnitrosamine-induced PSS and reported that the half-life of ICG is prolonged in these models.⁽³⁶⁾ Kim et al. reported that the sensitivity of PSS detection by CE-CT is 96%,⁽³⁷⁾ and we confirmed the absence of PSS in our experimental model after CCl₄ administration by CE-CT (Fig. 5). As a result, the prolonged half-life of ICG in this model reflected liver function. Although we observed a prolonged half-life of ICG and an increase in the fibrosis area in the control group, we also observed amelioration of fibrosis in the BMSC group. After 18 weeks of CCl₄ injections, exacerbation of liver fibrosis and increased ICG half-life were confirmed in both groups. Consequently, the duration of the effect of BMSCs in this canine liver fibrosis model with repeated CCl₄ injection is inferred to be about 4 weeks. In the future, further treatment effects are expected with repeated infusion of frozen BMSCs.

Biochemical examination of blood parameters and prothrombin time did not indicate significant worsening of liver fibrosis in our experimental model, and albumin and antithrombin 3 activities were improved in the BMSC group compared to the control group. Boothe et al. found small differences in clinical laboratory tests between canines with mild and severe dimethylnitrosamine-induced hepatic disease, and they suggested ICG may be an appropriate indicator of progressive changes in hepatic function because of its potential sensitivity.⁽²⁷⁾ Thus, we believe that the ICG test is useful for evaluation of liver function.

The expression of genes such as *COL1A2*, *COL3*, *TIMP-1*, *EDNRA*, and *EDNRB* was significantly lower in the BMSC group compared with the control group. Kanemoto et al. reported that the expression of these genes correlated well with the histologic degree of fibrosis in canines.⁽³⁸⁾ Additionally, in the cirrhotic rat liver, high expression of *EDNRA* and *EDNRB* was detected on hepatic stellate cells.⁽³⁹⁾ The results of the

present study showed that *EDNRA* and *EDNRB* expression was significantly lower in the BMSC group compared with the control group at 4W, which is consistent with the degree of fibrosis.

The mechanisms underlying BMSC-mediated improvement in fibrosis remain to be clarified. Several studies have reported that the antifibrotic effects of MSC therapy may be mediated by various types of trophic factors and cytokines produced by BMSCs.^(40,41) Most studies use the intravenous route, and a large proportion of MSCs that are injected through this route are trapped in the lungs upon first passage.⁽⁴²⁻⁴⁵⁾ Consequently, avoiding the lung trap may improve the survival of MSCs and may affect the distribution of the cells after administration. Arterial injection may ensure better delivery of MSCs to target organs. MSCs injected into the renal arteries are retained in the glomeruli for at least several days.⁽⁴⁶⁾ A study examining the engraftment of MSCs in the liver concluded that administration through the portal vein leads to far better engraftment than administration through the vena cava.⁽⁴⁷⁾ Based on these data, we expect that infusion of BMSCs through the hepatic artery with a hepatic angiography procedure will induce better regeneration of the fibrotic liver. It will be important to compare the therapeutic effects in this liver fibrosis model using different routes of administration of BMSCs.

In conclusion, we established a useful canine liver fibrosis model after repeated CCl₄ administration through a catheter for 10 weeks. Cultured autologous BMSC infusion improved liver fibrosis without adverse effects, raising the possibility of a less invasive therapy using cultured autologous BMSCs.

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